

Right Available Copy

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



Document FP1
Appl No To Be Assigned

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|--|--|----|---|
| (51) International Patent Classification 5 : G01N 33/569, C07K 15/28 | | A1 | (11) International Publication Number: WO 92/17785 (43) International Publication Date: 15 October 1992 (15.10.92) |
| (21) International Application Number: PCT/US92/02591 (22) International Filing Date: 30 March 1992 (30.03.92) | | | (81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), MC (European patent), NL (European patent), NO, SE (European patent). |
| (30) Priority data: 678,409 29 March 1991 (29.03.91) US | | | Published <i>With international search report.</i> |
| (71) Applicant: THE GENERAL HOSPITAL CORPORATION [US/US]; Office of Technology Affairs, Thirteenth Street, Building 149, Suite 1101, Charlestown, MA 02129 (US). | | | |
| (72) Inventor: MILLER, Samuel, I., III ; 144 Jordan Road, Brookline, MA 02146 (US). | | | |
| (74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US). | | | |

(54) Title: DETECTION OF SALMONELLA

(57) Abstract

A method of detecting *Salmonella* in a sample including contacting the sample with an antibody specific for a *PhoP* regulated gene product, allowing the antibody to form immune complexes with *Salmonella*, and detecting the immune complexes as an indication of the presence of *Salmonella* in the sample.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AT | Austria | ES | Spain | MG | Madagascar |
| AU | Australia | FI | Finland | ML | Mali |
| BB | Barbados | FR | France | MN | Mongolia |
| BE | Belgium | GA | Gabon | MR | Mauritania |
| BF | Burkina Faso | GB | United Kingdom | MW | Malawi |
| BG | Bulgaria | CN | Guinea | NL | Netherlands |
| BJ | Benin | GR | Greece | NO | Norway |
| BR | Brazil | HU | Hungary | PL | Poland |
| CA | Canada | IT | Italy | RO | Romania |
| CF | Central African Republic | JP | Japan | RU | Russian Federation |
| CG | Congo | KP | Democratic People's Republic of Korea | SD | Sudan |
| CH | Switzerland | KR | Republic of Korea | SE | Sweden |
| CI | Côte d'Ivoire | LI | Liechtenstein | SN | Senegal |
| CM | Cameroon | LK | Sri Lanka | SU | Soviet Union |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| DE | Germany | MC | Mongolia | TC | Togo |
| DK | Denmark | | | US | United States of America |

- 1 -

DETECTION OF SALMONELLA

The invention relates to the immunological detection of *Salmonella*.

5

Background of the Invention

This invention was made in the course of work supported by the United States Government, which has certain rights in the invention.

- Enteric fevers and diarrheal diseases, e.g.,
- 10 typhoid fever and cholera, are major causes of morbidity and mortality throughout the developing world, Hook et al., 1980, In Harrison's Principles of Internal Medicine, 9th Ed., 641-848, McGraw Hill, New York. *Salmonella* species cause a spectrum of clinical disease that
- 15 includes enteric fevers and acute gastroenteritis, Hook et al., 1980, *supra*.

S. typhi, the bacterium that causes typhoid fever, can only infect man. The narrow host specificity of *S. typhi* has resulted in the extensive use of *S. enteriditidis*

20 *typhimurium* infection of mice as a laboratory model of typhoid fever, Carter et al., 1984 J. Exp. Med. 159:1189. *S. typhimurium* infects a wider range of hosts, causing acute gastroenteritis in man and a disease similar to typhoid fever in the mouse and cow.

- 25 Recent studies have begun to define the molecular basis of *Salmonella typhimurium* virulence, Miller et al., 1989, Proc. Natl. Acad. Sci. USA 86:5054, hereby incorporated by reference. *Salmonella typhimurium* strains with mutations in the positive regulatory regulon
- 30 *phoP* are markedly attenuated in virulence for BALB/c mice. The *phoP* regulon is composed of two genes present in an operon, termed *phoP* and *phoQ*. The *phoP* and *phoQ* gene products are highly similar to other members of bacterial two-component transcriptional regulators that
- 35 respond to environmental stimuli and control the

- 2 -

expression of a large number of other genes. A mutation at one of these *phoP* regulatory region regulated genes, *pagC*, confers a virulence defect. Strains with *pagC*, *phoP*, or *phoQ* mutations afford partial protection to 5 subsequent challenge by wild-type *S. typhimurium*.

Summary of the Invention

In general the invention features a method of detecting *Salmonella* in a sample. The method includes contacting the sample with an antibody specific for a 10 *phoP* regulated gene product, e.g., the product of a *pag* gene, e.g., *pagC*, or a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgD*, *prgE*, *prgF*, *prgG*, or *prgH*; allowing the antibody to form immune complexes with *Salmonella*, and detecting the immune complexes as an indication of the presence of 15 *Salmonella* in the sample.

The invention also features a purified antibody, e.g., a monoclonal antibody, against a *phoP* regulated gene product, e.g., against a *pag* gene, e.g., *pagC*, or a *prg* gene, e.g., *prgA*, *prgB*, *prgC*, *prgD*, *prgE*, *prgF*, *prgG*, 20 or *prgH*.

Specific for a *phoP* regulated gene product, as used herein, refers to an antibody which binds to a *Salmonella phoP* regulated gene product but not to lysates of another gram negative bacterium, e.g., *E. coli*.

25 A purified preparation, as used herein, refers to an antibody preparation in which antibodies of the desired specificity constitute at least 50% (wt), and preferably at least 80% (wt), of the antibodies in the preparation.

30 The *phoP* regulatory region, as used herein, is a two-component regulatory system that controls the expression of *pag* and *prg* genes. It includes the *phoP* locus and the *phoQ* locus.

- 3 -

phoP regulatory region regulated genes, or *phoP* regulated genes, as used herein, refer to genes such as *pag* and *prg* genes.

pag, as used herein, refers to a gene which is
5 positively regulated by the *phoP* regulon.

prg, as used herein, refers to a gene which is negatively regulated by the *phoP* regulon.

phoP regulated genes are involved in virulence. Virulent bacteria generally contain genes which encode 10 virulence proteins specific to the organism. Therefore, the use of virulence gene products allow the detection of disease causing organisms without interference by or detection of comensal organisms. Because they interact with host organism structures, virulence proteins are 15 generally secreted or located on the membrane. Thus *phoP* regulated gene products are likely to posses two properties important to proteins used as targets for the immunological identification of a microorganism.

The invention provides a rapid and economic assay 20 for *Salmonella* in a variety of samples, including food, water, agricultural products, e.g., poultry, products, blood, urine, and feces.

Other features and advantages of the invention will be apparent from the following description of the 25 preferred embodiments and from the claims.

Description of the Preferred Embodiments

The drawings will first be described.

Drawings

Fig. 1 is a map of the restriction endonuclease 30 sites of the *pagC* locus.

Fig. 2 is a map of the DNA sequence of the *pag C* region (Sequence ID No. 1).

- 4 -

Strain Deposit

PhoP^C strain CS022 (described below) has been deposited with the American Type Culture Collection (Rockville, MD) and has received ATCC designation 10428.

5 The phoP system

The *phoP* regulatory region includes two regulatory genes, *phoP* and *phoQ*, and is essential for full virulence, survival within macrophages, and defensin resistance of *Salmonella typhimurium*. The *phoP* and *phoQ* proteins have amino acid similarity to the gene products of other bacterial two-component which control the synthesis of many proteins in response to environmental signals. The *PhoP* and *PhoQ* gene products are essential for the transcriptional activation of a number of unlinked *phoP*-activated genes, the *pag* loci. Another set of genes, the *prg* loci, are repressed by the wild type function of *PhoP*. As discussed below, a *phoP^C* (constitutive) mutation results in the constitutive induction of *pag* genes and the constitutive repression of *prg* genes.

The *phoP* constitutive allele (*PhoP^C*), *pho-24*, results in derepression of multiple *pag* loci.

Using diethyl sulfate mutagenesis of *S. typhimurium* LT-2, Ames and co-workers isolated strain TA2367 *pho-24* (all strains, materials, and methods referred to in this section are described below), which contained a *phoP* locus mutation that resulted in constitutive production of acid phosphatase in rich media, Kier et al., 1979, J. Bacteriol. 138:155, hereby incorporated by reference. This *phoP*-regulated acid phosphatase is encoded by the *phoN* gene, a *pag* locus, Kier et al., 1979, supra, Miller et al., 1989, supra. To analyze whether the *pho-24* allele increased the expression of other *pag* loci the effect of the *pho-24* allele on the expression of other *pag* loci recently

- 5 -

identified as transcriptional (e.g., *pagA* and *pagB*) and translational (e.g., *pagC*) fusion proteins that required *phoP* and *phoQ* for expression, Miller et al., 1989, supra, was determined. *pag* gene fusion strains, isogenic except 5 for the *pho-24* allele, were constructed and assayed for fusion protein activity. *PhoP*^c derivatives of the *pagA::Mu dJ* and *pagB::Mu dJ* strains produced 480 and 980 U, respectively, of β -galactosidase in rich medium, an increase of 9- to 10-fold over values for the fusion 10 strains with a wild-type *phoP* locus, see Table 1.

- 6 -

TABLE 1. Bacterial strains and properties

| Strain | Genotype | Enzyme activity (U) ^a | Reference or source |
|---------|---|----------------------------------|--|
| 10428 | Wild type | 180 (A) | ATCC; Miller et al., 1989, supra |
| TA2367 | <i>pho-24</i> | 1,925 (A) | Kier et al., 1974, supra |
| CS003 | $\Delta phoP \Delta purB$ | <10 (A) | Miller et al., 1989, supra |
| CS022 | <i>pho-24</i> | 1,750 (A) | This work |
| CS023 | <i>pho-24 phoN2</i> | 25 (A) | This work |
| CS012 | <i>zxx::6251Tn10d-Cam pagA1::MU dJ</i> | 45 (B) | Miller et al., 1989, supra |
| CS013 | <i>pagB1::MU dJ</i> | 120 (B) | Miller et al., 1989, supra |
| CS119 | <i>pagC1::TnphoA phoN2</i> | 85 (C) | Miller et al., 1989, supra |
| SC024 | <i>zxx::6251Tn10d-Cam pagA1::Mu dJ pho-24</i> | 450 (B) | This work |
| SC025 | <i>pagB1::Mu dJ pho-24</i> | 980 (B) | This work |
| SC026 | <i>pagC1::TnphoApho-24phoN2</i> | 385 (B) | This work |
| CS015 | <i>zxx::6251Tn10d-Cam phoP102::Tn10d-Cam</i> | <10 (A) | Miller et al., 1989, supra |
| TT13208 | <i>phoP105::Tn10d</i> | <10 (A) | -- ^b |

^a A. Acid phosphatase; B, β -galactosidase; C, alkaline phosphatase.

^b Gift of Ning Zhu and John Roth.

- 7 -

The *pagC::TnphoA* gene fusion produced 350 U of alkaline phosphatase, an increase of three- to fourfold over that produced in strain CS119, which is isogenic except for the *pho-24* mutation, Miller et al., 1989,
5 supra. These results compare with a ninefold increase in the acid phosphatase activity in strain CS022 on introduction of the *pho-24* allele. Therefore, these available assays for *pag* gene expression document that the *pho-24* mutation causes constitutive expression of *pag*
10 loci other than *phoN*.

The *phoP* constitutive allele results in repression of multiple *prg* loci. Whole-cell proteins of strain CS022 were analyzed to estimate the number of protein species that could be potentially regulated by the *PhoP*
15 regulon. Remarkably, analysis by one-dimensional polyacrylamide gel electrophoresis of the proteins produced by strains with the *PhoP^c* phenotype indicated that some protein species were decreased in expression when many presumptive *pag* gene products were fully
20 induced by the *pho-24* mutation. The proteins decreased in the *PhoP^c* strain might represent products of genes that are repressed by the *PhoP* regulator. Genes encoding proteins decreased by the *pho-24* allele are designated *prg* loci, for *phoP*-repressed genes. Comparison of wild-
25 type, *PhoP⁻*, and *PhoP^c* mutant strain proteins shows that growth in LB medium at 37°C represents repressing conditions for *pag* gene products and derepressing conditions for *prg* gene products.

To estimate the total number of potentially *PhoP*-regulated gene products, the total cell proteins of wild-type and *PhoP^c* mutant strains grown in LB were analyzed by two-dimensional gel electrophoresis. Approximately 40 species underwent major fluctuation in expression in response to the *pho-24* mutation.

- 8 -

Virulence defects of the *PhoP^c* strain Remarkably, strains with the single *pho-24* mutation were markedly attenuated for virulence in mice. The number of *PhoP^c* organisms (2×10^5) that killed 50% of BALB/c mice 5 challenged (LD_{50}) by the intraperitoneal (i.p.) route was near that (6×10^5) of *PhoP^c* bacteria, Miller et al., 1989, supra. The *PhoP^c* strains had growth comparable to wild-type organisms in rich and minimal media. The *PhoP^c* mutants were also tested for alterations in 10 lipopolysaccharide, which could explain the virulence defect observed. Strain CS022 had normal sensitivity to phage P22, normal group B reactivity to antibody to O antigen, and a lipopolysaccharide profile identical to that of the parent strain, as determined by 15 polyacrylamide gel electrophoresis and staining.

Strains, materials and methods The strains, materials, and methods used in the *PhoP* regulon work described above are as follows.

American Type Culture Collection (ATCC) strain 20 14028, a smooth virulent strain of *S. typhimurium*, was the parent strain for all virulence studies. Strain TT13208 was a gift from Nang Zhu and John Roth. Strain TA2367 was a generous gift of Gigi Stortz and Bruce Ames, Kier et al., 1979, supra. Bacteriophage P22HT int was 25 used in transductional crosses to construct strains isogenic except for *phoP* locus mutations, Davis et al., 1980, Advanced Bacterial Genetics, p. 78, 87. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, hereby incorporated by reference. Luria broth was used as rich 30 medium, and minimal medium was M9, Davis et al., 1980, supra. The chromogenic phosphatase substrate 5-bromo-4-chloro-3indolyl phosphate (XP) was used to qualitatively access acid and alkaline phosphatase production in solid media.

- 9 -

Derivatives of *S. typhimurium* ATCC 10428 with the *pho-24* mutation were constructed by use of strain TA2367 as a donor of the *purB* gene in a P22 transductional cross with strain CS003 Δ *phoP* Δ *purB*, Miller et al., 1989,
5 supra. Colonies were then selected for the ability to grow on minimal medium. A transductant designated CS022 (phenotype *PhoP^C*) that synthesized 1,750 U of acid phosphatase in rich medium (a ninefold increase over the wild-type level in rich medium) was used in further
10 studies.

Derivatives of strains CS022 and CS023 *pho-24* *phoN2* *zxx::6251Tn10d-Cam*, and acid phosphatase-negative derivative of CS022, containing *pag* gene fusions were constructed by bacteriophage P22 transductional crosses,
15 using selection of *TnphoA*- or *Mu dJ*-encoded kanamycin resistance. Strains were checked for the intact *pag* gene fusion by demonstration of appropriate loss of fusion protein activity on introduction of a *phoP105::Tn10d* or *phoP102::Tn10d-Cam* allele.

20 Assays of acid phosphatase, alkaline phosphatase, and β -galactosidase were performed as previously described, Miller et al., 1989, supra and are reported in units as defined in Miller, 1972, Experiments in molecular genetics, p. 352-355, Cold Spring Harbor
25 Laboratory, Cold Spring Harbor, NY, hereby incorporated by reference.

In the mouse virulence and vaccination studies bacteria grown overnight in Luria broth were washed and diluted in normal saline. The wild-type parent strain of
30 CS022 (ATCC 10428) was used for all live vaccine challenge studies. This strain has a 50% lethal dose (LD_{50}) for naive adult BALB/c mice of less than 20 organisms when administered by intraperitoneal (i.p.) injection and 5×10^4 when administered orally in NaHCO₃.
35 Mice were purchased from Charles River Breeding

- 10 -

Laboratories, Inc. (Wilmington, Mass.) and were 5 to 6 weeks of age at initial challenge. All i.p. inoculations were performed as previously described, Miller et al., 1989, supra. Oral challenge experiments were performed 5 with bacteria grown in LB broth and concentrated by centrifugation. The bacteria were resuspended in 0.1 M NaHCO₃ to neutralize stomach acid, and administered as a 0.5-ml bolus to animals under ether anesthesia. Colony counts were performed to accurately access the number of 10 organisms administered. All challenge experiments were performed 1 month after i.p. inoculation and 6 weeks after oral challenge. Challenge inocula were administered by the same route as vaccinations. The care of all animals was under institutional guidelines as set 15 by the animal care committees at the Massachusetts General Hospital and Harvard Medical School.

Protein electrophoresis was performed as follows. One-dimensional protein gel electrophoresis was performed by the method of Laemmli, 1970, Nature 227:680, hereby 20 incorporated by reference, on whole-cell protein extracts of stationary-phase cells grown overnight in Luria broth. The gels were fixed and stained with Coomassie brilliant blue R250 in 10% acetic acid-10% methanol. Two-dimensional protein gel electrophoresis was performed by 25 method of O'Farrell, 1975, J. Biol. Chem. 250:4007, hereby incorporated by reference, on the same whole-cell extracts. Isoelectric focusing using 1.5% pH 3.5 to 10 ampholines (LKB Instruments, Baltimore, Md.) was carried out for 9,600 V h (700 V for 13 h 45 min). The final 30 tube gel pH gradient extended from pH 4.1 to pH 8.1 as measured by a surface pH electrode (BioRad Laboratories, Richmond, Calif.) and colored acetylated cytochrome pI markers (Calbiochem-Behring, La Jolla, Calif.) run in an adjacent tube. The slab gels were silver stained, Merril

- 11 -

et al., 1984, Methods Enzymol. 104:441, hereby incorporated by reference.

The isolation of phoP regulated genes

PhoP regulated genes (i.e., pag and prg genes) can 5 be identified and cloned by mutagenizing a *Salmonella* strain with a transposon which carries a marker gene, e.g., the lacZ gene, or a gene encoding alkaline phosphatase, and screening for phoP regulated expression of the transposon-borne marker. phoP regulated 10 expression can be identified by comparing the expression of the inserted marker gene in wild type and in phoP regulatory region mutant backgrounds.

The expression of pag genes is positively regulated by PhoP, thus an insertion into a pag will be 15 characterized by relatively high expression in a PhoP⁺ or PhoP^c background, and relatively low expression in a PhoP⁻ background, on rich medium. The expression of prg genes is negatively regulated by PhoP. Thus, an insertion of a transposon borne marker into a prg gene will be 20 characterized by relatively low expression in a PhoP⁺ or PhoP^c background, and by relatively high expression in a PhoP⁻ background, on rich medium.

Once insertions at putative prg and pag loci are identified the insertionally disrupted genes can be 25 cloned and used to identify and clone wild type genomic DNA corresponding to the site of the insertional mutation.

This method of isolating pag and prg genes is described in Miller et al., 1989, *supra*, and herein.

- 12 -

The pagC gene and pagC gene product

Strains, materials, and methods The following strains, materials, and methods were used in the cloning of pagC and in the analysis of the gene and its gene product.

Rich media was Luria broth (LB) and minimal media was M9, Davis et al., 1980, supra. The construction of *S. typhimurium* strain CS119 pagC1::TnphoA phoN2 zxx::6251 Tn10d-Cam was previously described, Miller et al., 1989, supra. American Type Culture Collection (ATCC) *S. typhimurium* strain 10428 included CS018 which is isogenic to CS119 except for phoP105::Tn10d, Miller et al., 1989, supra, CS022 pho-24, Miller et al., 1990, J. Bacteriol. 172:2485-2490, hereby incorporated by reference, and CS015 phoP102::Tn10d-cam, Miller et al., 1989, supra. Other wild type strains used for preparation of chromosomal DNA included *S. typhimurium* LT2 (ATCC 15277), *S. typhimurium* Q1 and *S. drypool* (Dr. J. Peterson U. Texas Medical Branch, Galveston), and *Salmonella typhi* Ty2 (Dr. Caroline Hardegree, Food and Drug Administration). pLAFR cosmids were mobilized from *E. coli* to *S. typhimurium* using the *E. coli* strain MM294 containing pRK2013, Friedman et al., 1982, Gene 18:289-296, hereby incorporated by reference. Alkaline phosphatase (AP) activity was screened on solid media using the chromogenic phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate (XP). AP assays were performed as previously described, Brickman et al., 1975, J. Mol. Biol. 96:307-316, hereby incorporated by reference, and are reported in units as defined by Miller, Miller, 1972, supra, pp. 352-355.

One dimensional protein gel electrophoresis was performed by the method of Laemmli, 1970, Nature, 227:680-685, hereby incorporated by reference, and blot hybridization using antibody to AP was performed as

- 13 -

previously described, Peterson et al., 1988, Infect. Immun. 56:2822-2829, hereby incorporated by reference. Whole cell protein extracts were prepared, from saturated cultures grown in LB at 37°C with aeration, by boiling 5 the cells in SDS-pagE sample buffer, Laemmli, 1970, supra. Two dimensional gel electrophoresis was performed by the method of O'Farrell, 1975, J. Biol. Chem. 250:4007, hereby incorporated by reference. Proteins in the 10% polyacrylamide slab gels were visualized by 10 silver staining, Merril et al., 1984, Methods in Enzymology, 104:441, hereby incorporated by reference. Chromosomal DNA was prepared by the method of Mekalanos, 1983, Cell, 35:253-263, hereby incorporated by reference. DNA, size fractionated in agarose gels, was 15 transferred to nitrocellulose (for blot hybridization) by the method of Southern, 1975, J. Mol. Biol. 98:503-517, hereby incorporated by reference. DNA probes for Southern hybridization analysis were radiolabeled by the random primer method, Frinberg et al., 1984, supra. 20 Plasmid DNA was transformed into *E. coli* and *Salmonella* by calcium chloride and heart shock, Mekalanos, 1983, supra, or by electroporation using a Genepulser apparatus (Biorad, Richmond, Ca.) as recommended by the manufacturer, Dower et al., 1988, Nucl. Acids Res. 25 16:6127-6145, hereby incorporated by reference. DNA sequencing was performed by the dideoxy chain termination method of Sanger et al., 1977, Proc. Natl. Acad. Sci. USA, 74:5463-5467, hereby incorporated by reference, as modified for use with Sequenase (U.S. Biochemical, 30 Cleveland, Ohio). Oligonucleotides were synthesized on an Applied Biosystems Machine and used as primers for sequencing reactions and primer extension of RNA. Specific primers unique to the two ends of TnphoA, one of which corresponds to the alkaline phosphatase coding 35 sequence and the other to the right IS50 sequence, were

- 14 -

used to sequence the junctions of the transposon insertion.

Construction of a *S. typhimurium* cosmid gene bank in pLAFR3 and screening for clones containing the wild type pagC DNA was performed as follows. DNA from *S. typhimurium* strain ATCC 10428 was partially digested using the restriction endonuclease Sau3A and then size selected on 10-40% sucrose density gradient. T4 DNA ligase was used to ligate chromosomal DNA of size 20-30 kilobases into the cosmid vector pLAFR3, a derivative of pLAFR1, Friedman et al., 1982, Gene 18:289-296, hereby incorporated by reference, that was digested with the restriction endonuclease BamHI. Cosmid DNA was packaged and transfected into *E. coli* strain DH5- α using extracts purchased from Stratagene, La Jolla, Ca. Colonies were screened by blot hybridization analysis.

The analysis of proteins produced from cloned DNA by in vitro transcription/translation assays was analyzed as follows. These assays were performed with cell free extracts, (Amersham, Arlington Heights, Illinois), and were performed using conditions as described by the manufacturer. The resultant radiolabeled proteins were analyzed by SDS-page.

RNA was purified from early log and stationary phase *Salmonella* cultures by the hot phenol method, Case et al., 1988, Gene 72:219-236, hereby incorporated by reference, and run in agarose-formaldehyde gels for blot hybridization analysis, Thomas, 1980, Proc. Natl. Acad. Sci. USA 77:5201, hereby incorporated by reference. Primer extension analysis of RNA was performed as previously described, Miller et al., 1986, Nuc. Acids. Res. 14:7341-7360, hereby incorporated by reference, using AMV reverse transcriptase (Promega, Madison, Wisconsin) and synthesized oligonucleotide primers

- 15 -

complementary to nucleotides 335-350 and 550-565 of the pagC locus.

Construction of a pagC insertional mutation pagC
was identified by the insertion of TnphoA. TnphoA is a
5 transposon which carries the marker alkaline phosphatase.
A pagC::TnphoA insertion in a phoP⁺ background had a 20
fold increase in expression of the marker as compared to
a pagC::TnphoA insertion in a phoP deletion background.
See Miller et al., 1989, *supra*.

10 Random transposon mutagenesis of *S. typhimurium*
was performed as described in Miller et al., 1989, *supra*,
by using MudJ, described in Hughes et al., 1988, Mol.
Gen. Genet. 119:9, hereby incorporated by reference,
Tn10d-Cam, described in Eliot et al., 1988 Mol. Gen.
15 Genet. 213:332, hereby incorporated by reference, and
Bender et al., 1986, Cell 45:801, and TnphoA, described
in Taylor et al., 1989, J. Bact. 171:1870, hereby
incorporated by reference. *Escherichia coli* strain CS118
was used as a phoA-negative recipient for TnphoA
20 mutagenesis of plasmid DNA with TnphoA as described in
Gutierrez et al., 1987, J. Mol. Biol. 195:289, hereby
incorporated by reference.

Identification of an 18 kDa protein missing in a
pagC mutant of S. typhimurium pagC mutant strain CS119
25 was analyzed by two dimensional protein electrophoresis
to detect protein species that might be absent as a
result of the TnphoA insertion. Only a single missing
protein species, of approximately 18 kD and pI-8.0, was
observed when strains, isogenic except for their
30 transposon insertions, were subjected to this analysis.
This 18 kDa species was also missing in similar analysis
of *Salmonella* strains with mutations phoP and phoQ.
Though two-dimensional protein gel analysis might not
detect subtle changes of protein expression in strain

- 16 -

CS119, this suggested that a single major protein species was absent as a result of the *pagC::TnphoA* insertion.

Additional examination of the 2-dimensional gel analysis revealed a new protein species of about 45 kDa
5 that is likely the *pagC*-Ap fusion protein. The *pagC*-AP fusion protein was also analyzed by Western blot analysis using antisera to AP and found to be similar in size to native AP (45 kDa) and not expressed in *PhoP-S. typhimurium*.

10 Cloning of the *pagC::TnphoA* insertion Chromosomal DNA was prepared from *S. typhimurium* strain CS119 and a rough physical map of the restriction endonuclease sites in the region of the *pagC::TnphoA* fusion was determined by using a DNA fragment of *TnphoA* as a probe in blot
15 hybridization analysis. This work indicated that digestion with the restriction endonuclease *ecoRV* yielded a single DNA fragment that included the *pagC::TnphoA* insertion in addition to several kilobases of flanking DNA. Chromosomal DNA from strain CS119 was digested with
20 *EcoRV* (blunt end) and ligated into the bacterial plasmid vector pUC19 (New England Biolabs) that had been digested with the restriction endonuclease *SmaI* (blunt end). This DNA was electroporated into the *E. coli* strain DH5- α (BRL) and colonies were plated onto LB agar containing
25 the antibiotics kanamycin (*TnphoA* encoded) and ampicillin (pUC19 encoded). A single ampicillin and kanamycin resistant clone containing a plasmid designated pSM100 was selected for further study.

A radiolabeled DNA probe from pSM100 was
30 constructed and used in Southern hybridization analysis of strain CS119 and its wild type parent ATCC 10428 to prove that the *pagC::TnphoA* fusion had been cloned. The probe contained sequences immediately adjacent to the transposon at the opposite end of the alkaline
35 phosphatase gene [*HpaI* endonuclease generated DNA

- 17 -

fragment that included 186 bases of the right IS50 of the transposon and 1278 bases of *Salmonella* DNA (Fig. 1). As expected, the pSM100 derived probe hybridized to an 11-12 kb *AccI* endonuclease digested DNA fragment from the 5 strain containing the transposon insertion, CS119. This was approximately 7.7kb (size of TnphoA) larger than the 3.9 kB *AccI* fragment present in the wild type strain that hybridizes to the probe. In addition, a derivative of plasmid pSM100, pSM101 (which did not allow expression of 10 the *pagC*-*PhoA* gene fusion off the *lac* promoter), was transformed into *phoP-* (strain Cs015) and *phoN-* (strain CS019) *Salmonella* strains and the cloned AP activity was found to be dependent on *phoP* for expression. Therefore we concluded that the cloned DNA contained the 15 *pagC::TnphoA* fusion.

Cloning of the wild type *pagC* locus DNA and its complementation of the virulence defect of a *S. typhimurium* *pagC* mutant The same restriction endonuclease fragment described above was used to screen 20 a cosmid gene bank of wild type strain ATCC 10428. A single clone, designated pWP061, contained 18 kilobases of *S. typhimurium* DNA and hybridized strongly to the *pagC* DNA probe. pWP061 was found to contain *Salmonella* DNA identical to that of pSM100 when analyzed by restriction 25 endonuclease analysis and DNA blot hybridization studies. Probes derived from pWP061 were also used in blot hybridization analysis with DNA from wild type and CS119 *S. typhimurium*. Identical hybridization patterns were observed to those seen with pSM100. pWP061 was also 30 mobilized into strain CS119, a *pagC* mutant strain. The resulting strain had wild type virulence for BALB/c mice (a LD₅₀ less than 20 organisms when administered by IP injection). Therefore the cloned DNA complements the virulence defect of a *pagC* mutant strain.

- 18 -

Since, a wild type cosmid containing *pagC* locus DNA was found to complement the virulence defect of a *pagC* mutant *S. typhimurium* strain, it was concluded that the *pagC* protein is an 188 amino acid (18 kDa) membrane 5 (see below) protein essential for survival within microphages and virulence of *S. typhimurium*.

Physical mapping of restriction endonuclease sites, DNA sequencing, and determination of the *pagC* gene product Restriction endonuclease analysis of plasmid 10 pSM100 and pWP061 was performed to obtain a physical map of the *pagC* locus, and, in the case of PSM100, to determine the direction of transcription (Fig. 1). DNA subclones were generated and the TnphoA fusion junctions were sequenced, as well as the *Salmonella* DNA extending 15 from the *Hpa*I site, 828 nucleotides 5' to the *phoA* fusion junction, to the *Eco*RI site 1032 nucleotides 3' to the TnphoA insertion (Fig. 1 and 2). The correct reading frame of the DNA sequence was deduced from that required to synthesize an active AP gene fusion. The deduced 20 amino acid sequence of this open reading frame was predicted to encode a 188 amino acid protein with a predicted pI+8.2. This data were consistent with the 2-D polyacrylamide gel analysis of strain CS119 in which an 18 kDa protein of approximate pI+8.0 was absent. No 25 other open reading frames, predicted to encode peptides larger than 30 amino acids, were found.

The deduced amino acid sequence of the 188 amino acid open reading frame contains a methionine start codon 33 amino acids from the fusion of *pagC* and AP (Fig. 2). 30 This 33 amino acid *pagC* contribution to the fusion protein was consistent with the size observed in Western blot analysis and contains a hydrophobic N-terminal region, identified by the method of Kyle et al., 1982, J. Mol. Biol. 157:105-132, hereby incorporated by reference, 35 that is a typical bacterial signal sequence, Von Heinje,

- 19 -

1985, J. Mol. Biol. 184:99-105, hereby incorporated by reference. Specifically, amino acid 2 is a positively charged lysine, followed by a hydrophobic domain and amino acid 24 is a negatively charged aspartate residue.

5 A consensus cleavage site for this leader peptide is predicted to be at an alanine residue at amino acid 23, Von Heinje, 1984, J. Mol. Biol. 173:243-251, hereby incorporated by reference. The DNA sequence also revealed a typical ribosomal binding site, Shine et al.,

10 1974, Proc. Natl. Acad. Sci. USA 71:1342-1346, hereby incorporated by reference, at 6-2 nucleotides 5' to the predicted start of translation (Fig. 2) nucleotides 717-723). This suggested that the open reading frame was, in fact, translated and further supported the assumption

15 that this was the deduced amino acid sequence of the pagC protein interrupted by the TnphoA insertion (Fig. 2).

In vitro synthesis of proteins by the cloned pagC locus To detect if other proteins were encoded by pagC and to determine the approximate size of the pagC gene product, an *in vitro* coupled transcription/translation analysis was performed. A 5.3 kilobase EcoRI fragment of pWP061 was inserted into pUC19 so that the pagC gene would not be expressed off the lac promotor. This plasmid was used in an *in vitro* coupled transcription-translation assay. A single protein of approximately 22 kilodaltons was synthesized by the cell free system. The size was compatible with this being the precursor of the pagC protein containing its leader peptide. These data further support the conclusion the single and the single 30 pagC gene product had been identified.

Identification of the pagC encoded RNA An approximately 1100 nucleotide RNA is encoded by pagC. The pagC gene is highly expressed by cells with a *phoP* constitutive phenotype of *pag* activation, as compared to 35 wild type and *phoP* constitutive phenotype of *pag*

- 20 -

- activation, as compared to wild type and *phoP*- bacteria. In these blot hybridization experiments *pagC* is only detected in wild type cells grown in rich media during stationary growth. This result, coupled with previous work, Miller et al., 1989, supra, Miller et al., 1990, supra, demonstrates that *pagC* is transcriptionally regulated by the *phoP* gene products and is only expressed during early logarithmic phase growth in rich media by cells with a *phoP* constitutive phenotype.
- The size of the *pagC* transcript is approximately 500 nucleotides greater than that necessary to encode the 188 amino acid protein. Primer extension analysis of *Salmonella* RNA using oligonucleotide primers specific for *pagC* sequence was performed to determine the approximate start site of transcription and to determine whether these nucleotides might be transcribed 5' or 3' to the 188 amino acid *pagC* gene product. Primer extension analysis with an oligonucleotide predicted to be complementary to nucleotides 550-565 of *pagC*, 150 nucleotides 5' to the predicted start codon, resulted in an approximately 300 nucleotide primer extension product. Therefore a primer further upstream was constructed complementary to nucleotides 335-350 of *pagC* and used in a similar analysis. A primer extension product of 180 nucleotides was observed to be primer specific. This is consistent with transcription starting at nucleotide 170 (Fig. 2). Upstream of the predicted transcriptional start, at nucleotides 153-160, a classic RNA polymerase binding site was observed with the sequence TATAAT at -12 nucleotides as well as the sequence TAATAT at -10 nucleotides. No complete matches were observed for the consensus RNA polymerase recognition site (TTGACA) 15-21 nucleotides upstream from the -10 region. AT -39 (126-131) nucleotides (TTGGAA), -38 (127-132) nucleotides (TTGTGG), and -25 (135-140) nucleotides (TTGATT) are

- 21 -

sequences that have matches with the most frequently conserved nucleotides of this sequence.

Based on the above results transcription was predicted to terminate near the translational stop codon 5 of the 188 amino acid protein (nucleotide 1295, Fig. 2). Indeed, a stem loop configuration was found at nucleotides 1309-1330 that may function as a transcription terminator. This was consistent with the lack of evidence of open reading frames downstream of the 10 188 amino acid protein and the lack of synthesis of other transcription/translation using the cloned pagC DNA. This further suggests that the pagC::TnphoA insertion inactivated the synthesis of only a single protein.

pagC Mutant Strains Are Attenuated For Virulence

15 *Salmonella typhimurium* strains with a pagC mutation are most likely inactivated for the phoP-regulated gene product, as these strains are attenuated for virulence by at least 1,000-fold.

20 pagC is common to many *Salmonella* strains but lacking in many other bacteria. The presence of the pagC gene was also demonstrated in other strains of *S. typhimurium*, as well as in *S. typhi*, and *S. drypool*. All *Salmonella* strains examined demonstrated similar strong hybridization to an 8.0 kb EcoRV and a 3.9 kb AccI 25 restriction endonuclease fragment suggesting that pagC is a virulence gene common to *Salmonella* species.

The pagC gene probe from nucleotides -46 (with 1 as the first base of the methionine to 802 (PstI site to the BglII site) failed to cross hybridize to DNA from 30 *Citrobacter freundii*, *Shigella flexneri*, *Shigella sonnei*, *Shigella dysenteriae*, *Escherichia coli*, *Vibrio cholerae*, *Vibrio vulnificus*, *Yersenia enterocolitica*, and *Klebsiella pneumoniae*.

35 Lipopolysaccharide patterns and p22 bacteriophage sensitivity appear to be the same in phoP⁻, phoP^c, and

- 22 -

phoP⁺, bacgrounds, suggesting that *phoP* regulated genes do not encode flagellar or lipopolysaccharide flagellar or conventional lipopolysaccharide antigen products. Additionally, *phoP* mutants are motile, suggesting that 5 *phoP* regulated genes do not encode flagellar antigens.

Production of antibodies to the products of *phoP* regulatory region regulated genes

Antibodies to the products of the *phoP* regulatory region-regulated genes can be made by methods known to 10 those in the art. Polyclonal or monoclonal antibodies can be used in the methods of the invention.

Antibodies can be generated by challenge with any antigen which results in the production of antibodies which bind to a *pag* or *prg* gene product and which are 15 specific for *Salmonella*. Antibodies to a given *pag* or *prg* gene product can be produced, e.g., by challenge with *Salmonella* lysates; a naturally occurring example of *pag* or *prg* gene product, or fragments thereof; recombinant examples of *pag* or *prg* gene product, or fragments 20 thereof; synthetic *pag* or *prg* gene product, or fragments or homologs thereof; and in general, peptides which, regardless of their origin, length, or degree of exact or partial homology with a naturally occurring *pag* or *prg* gene product, produce an antibody which binds 25 specifically to the *pag* or *prg* gene product of interest.

Antibodies thus produced, whether polyclonal or monoclonal, are screened for suitability by the ability to bind to an epitope present on the naturally occurring *pag* or *prg* gene product in question.

30 Antibodies can be tested for specificity to *Salmonella* by methods known to those skilled in the art. For example an antibody preparation which, under a given set of conditions, binds to Western blots of *Salmonella* lysates but which fails to bind to Western blots of 35 lysates of other gram negative bacteria expected to be

- 23 -

present in the sample to be tested, e.g., *E. coli.*, are specific for *Salmonella*.

Anti-pagC antibodies

The peptide Arg-Gly-Val-Asn-Val-Lys-Tyr-Arg-Tyr-

- 5 Glu-Asp-Asp-Ser-Phe (Sequence No. 2) (the "pagC fragment"), which corresponds to amino acid residues 47-60 of the predicted pagC gene product of *S. typhimurium*, was produced by solid state synthesis, by methods known to those skilled in the art. The pagC fragment was
10 conjugated to keyhole limpet hemocyanin (KLH), by methods known to those skilled in the art, and injected into rabbits. Polyclonal antibodies were recovered and absorbed against a crude lysate of *E. coli*. This step was necessary because essentially all sera from rabbits
15 contain antibodies reactive with *E. coli*. The serum was tested prior to absorption and found to exhibit a high titre against the pagC fragment. The post-absorbed antiserum bound to Western blots of *Salmonella* lysates but not to Western blots of *E. coli* lysates. The
20 difference in signal strength between the *E. coli* blot and the *Salmonella* blot appeared to be at least 100 fold.

- The anti-pagC antiserum was used to demonstrate that pagC is an outer membrane protein. Fractions of *Salmonella* outer membranes, and of total *Salmonella* protein minus outer membranes, were prepared by methods known to those skilled in the art. Western blots of each fraction were probed with the anti-pagC fragment antibody. All signal was confined to the outer membrane blot.

30 Anti-prg gene product antibodies

TnphoA transposon insertions were used (as described above) to identify and recover several prg genes. TnphoA insertions identify envelop or membrane proteins because the marker encoded by TnphoA, alkaline

- 24 -

phosphatase, must dimerize in order to be active. Dimerization requires passage through the periplasmic space, and thus the presence of active alkaline phosphatase indicates insertion into a gene which encodes 5 a secreted, periplasmic, or membrane protein.

A prg gene product, or a fragment or homolog thereof, can be used to stimulate the production of anti-prg antibodies. Antibodies are selected as described above.

10 Monoclonal antibodies to the products of phop regulated genes

Monoclonal antibody are prepared by fusing spleen cells from a mammal which has been immunized against a pag or prg encoded antigen, with an appropriate myeloma 15 cell line. The resultant product is cultured in a standard HAT (hypoxanthine, aminopterin and thymidine) medium to yield hybridomas.

The immunized spleen cells may be derived from any mammal, e.g., mice. The animal is first immunized by 20 injection of the chosen antigen. When the animal shows sufficient antibody production against the antigen, as determined by conventional assay methods, it is given a booster injection of the antigen, and then killed so that the immunized spleen may be removed. The fusion can then 25 be carried out utilizing immunized spleen cells and an appropriate myeloma cell line.

Hybridomas are selected by screening for those which produce antibodies which bind to a molecule carrying the epitope against which they were generated, 30 e.g., in the case of a the pagC fragment, hybridomas could be screened with the pagC fragment peptide or with all or an appropriate portion of naturally occurring or recombinant pagC protein.

Detection of Salmonella

- 25 -

- The formation of an immune complex between antibodies against the products of *phoP* regulated genes and their target antigens can be used to detect the presence of *Salmonella* in a sample. A wide variety of 5 suitable methods for detecting the formation of immune complexes, including liquid phase and solid phase assays, are known to those skilled in the art and any of them can be used in conjunction with the antibodies of the invention for the detection of *Salmonella*.
- 10 Immunoassays are commonly carried out, at least in part, on solid supports, e.g., glass fiber membranes. Two of the most common forms of immunoassay which employ solid supports are competitive and sandwich formats. Typical competitive formats are described e.g., in
- 15 Littman et al., U.S. Patent 4,540,659, hereby incorporated by reference; and a typical sandwich assay by David et al., U.S. Patent 4,376,110, hereby incorporated by reference.
- In a sandwich assay the anti-*phoP* gene product 20 antibody can be bound to a solid support, contacted with the sample to be tested, the bound antibody and sample allowed to incubate, excess sample removed, and the antigen-antibody complexes contacted with a second antibody which binds to an epitope on the antigen (or on 25 the *Salmonella* cell carrying the antigen) other than that recognized by the anti-*phoP* gene product antibody. The epitope recognized by the second antibody need not be on the *pagC* gene product but may be any epitope on the surface of the bound *Salmonella* cell.
- 30 Other embodiments are with the following claims.
What is claimed is:

- 26 -

COMPUTER SUBMISSION OF DNA AND AMINO ACID SEQUENCES

(1) GENERAL INFORMATION:

(i) APPLICANT: Miller, Samuel I.

(ii) TITLE OF INVENTION: Detection of Salmonella

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Fish & Richardson
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: Massachusetts
(E) COUNTRY: U.S.A.
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb storage
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: IBM P.C. DOS (Version 3.30)
(D) SOFTWARE: WordPerfect (Version 5.0)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: 07/678,409
(B) FILING DATE: 29 March 1991

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Clark, Paul T.
(B) REGISTRATION NUMBER: 30,162
(C) REFERENCE/DOCKET NUMBER: 00786/084001

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (617) 542-5070
(B) TELEFAX: (617) 542-8906
(C) TELEX: 200154

- 27 -

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

| | |
|-------------------|--------------|
| (A) LENGTH: | 2320 |
| (B) TYPE: | nucleic acid |
| (C) STRANDEDNESS: | single |
| (D) TOPOLOGY: | linear |

(xi) SEQUENCE DESCRIPTION: SEQUENCE ID NO: 1:

| | |
|---|-----|
| GTAAACCACT CTTAATAATA ATGGGTTTTA TACCGAAATA CACTTTTTA TCGCGTGTTC | 60 |
| AATATTTGCG TTAGTTATTA TTTTTTGGA ATGTAATTTC TCTCTAAACA CAGGTGATAT | 120 |
| TTATGTTGGA ATTGTGGTGT TGATTCTATT CTTATAATAT ACAAGAAAAT GTTGTAACTG | 180 |
| ATAGATATAT TAAAAGATTA AATCGGAGGG GGAATAAACG GTGCTAAGCA TCATCGTGAA | 240 |
| TATGATTACA GCGCCTGCGA TGGCATATAA CCGTATTGCG GATGGAGCGT CACGTGAGGA | 300 |
| CTGTGAAGCA CAATGCGATA TGTTCTGATT ATATGGCGAG TTTGCTTAAT GACATGTTTT | 360 |
| TAGCCGAACG GTGTCAGTT TCTTAATGTG GTTGTGAGAT TTTCTTTTA AATATCAAAA | 420 |
| TGTTGCATGG GTGATTTGTT GTTCTATAGT GGCTAAAGAC TTTATGGTTT CTGTTAAATA | 480 |
| TATATGCGTG AGAAAATTA GCATTCAAAT CTATAAAAGT TAGATGACAT TGTAGAACCG | 540 |
| GTTACCTAAA TGAGCGATAG AGTGCTTCGG TAGAAAAAT ATCTTCAGG AAGTAAACAC | 600 |
| ATCAGGAGCG ATAGCGGTGA ATTATTCGTG GTTTGTCGA TTCGGCATAG TGGCGATAAAC | 660 |
| TGAATGCCGG ATCGGTACTG CAGGTGTTTA AACACACCGT AAATAATAAG TAGTATTAAG | 720 |
| GAGTTGTT | 728 |
| ATG AAA AAT ATT ATT TTA TCC ACT TTA GTT ATT ACT ACA AGC GTT TTG | 776 |
| Met Lys Asn Ile Ile Leu Ser Thr Leu Val Ile Thr Thr Ser Val Leu | |
| 5 10 15 | |
| Val Val Asn Val Ala Gln Ala Asp Thr Asn Ala Phe Ser Val Gly Tyr | |
| 20 25 30 | |
| GTT GTA AAT GTT GCA CAG GCC GAT ACT AAC GCC TTT TCC GTG GGG TAT | 824 |
| Val Val Asn Val Ala Gln Ala Asp Thr Asn Ala Phe Ser Val Gly Tyr | |
| 35 40 45 | |
| GCA CGG TAT GCA CAA AGT AAA GTT CAG GAT TTC AAA AAT ATC CGA GGG | 872 |
| Ala Arg Tyr Ala Gln Ser Lys Val Gln Asp Phe Lys Asn Ile Arg Gly | |

- 28 -

| | | |
|--|--|------|
| GTA AAT GTG AAA TAC CGT TAT GAG GAT GAC TCT CCG GTA AGT TTT ATT Val Asn Val Lys Tyr Arg Tyr Glu Asp Asp Ser Pro Val Ser Phe Ile | 50 55 60 | 920 |
| TCC TCG CTA AGT TAC TTA TAT GGA GAC AGA CAG GCT TCC GGG TCT GTT Ser Ser Leu Ser Tyr Leu Tyr Gly Asp Arg Gln Ala Ser Gly Ser Val | 65 70 75 80 | 968 |
| GAG CCT GAA GGT ATT CAT TAC CAT GAC AAG TTT GAG GTG AAG TAC GGT Glu Pro Glu Gly Ile His Tyr His Asp Lys Phe Glu Val Lys Try Gly | 85 90 95 | 1016 |
| TCT TTA ATG GTT GGG CCA GCC TAT CGA TTG TCT GAC AAT TTT TCG TTA Ser Leu Met Val Gly Pro Ala Tyr Arg Leu Ser Asp Asn Phe Ser Leu | 100 105 110 | 1064 |
| TAC GCG CTG GCG GGT GTC GGC ACG GTA AAG GCG ACA TTT AAA GAA CAT Tyr Ala Leu Ala Gly Val Gly Thr Val Lys Ala Thr Phe Lys Glu His | 115 120 125 | 1112 |
| TCC ACT CAG GAT GGC GAT TCT TTT TCT AAC AAA ATT TCC TCA AGG AAA Ser Thr Gln Asp Gly Asp Ser Phe Ser Asn Lys Ile Ser Ser Arg Lys | 130 135 140 | 1160 |
| ACG GGA TTT CCC TGG GGC GCG GGT GTC CAG ATG AAT CCG CTG GAG AAT Thr Gly Phe Ala Trp Gly Ala Gly Val Gln Met Asn Pro Leu Glu Asn | 145 150 155 160 | 1208 |
| ATC GTC GTC GAT GTT GGG TAT GAA GGA AGC AAC ATC TCC TCT ACA AAA Ile Val Val Asp Val Gly Tyr Glu Gly Ser Asn Ile Ser Ser Thr Lys | 165 170 175 | 1256 |
| ATA AAC GGC TTC AAC GTC GGG GTT GGA TAC CGT TTC TGA AAAGC Ile Asn Gly Phe Asn Val Gly Val Gly Tyr Arg Phe | 180 185 | 1300 |
| ATAAGCTATG CGGAAGGTTG GCCTTCGCA CCGCCAGTCA ATAAAACAGG GCTTCTTTAC | 1360 | |
| CAGTGACACG TACCTGCCCTG TCTTTCTCT CTTCGTCTATA CTCTCTTCGT CATAGTGACG | 1420 | |
| CTGTACATAA CATCTCACTA GCATAAGCAC AGATAAAGGA TTGTGGTAAG CAATCAAGGT | 1480 | |
| TGCTCAGGTA GGTGATAAGC AGGAAGGAAA ATCTGGTGTAAATAACGCCA GATCTCACAA | 1540 | |
| GATTCACTCT GAAAAATTTT CCTGGAATTAA ATCACAATGT CATCAAGATT TTGTGACCGC | 1600 | |
| CTTCGCATAT TGTACCTGCC GCTGAACGAC TACTGAAAAG TAGCAAGGTA TGTATTTAT | 1660 | |
| CCAGGAGAGC ACCTTTTTG CGCCTGGCAG AAGTCCCCAG CCGCCACTAG CTCAGCTGGA | 1720 | |
| TAGAGCATCA ACCTCCTAAG TTGATGGTGC GAGGTTCGAG GCCTCGGTGG CGGTCCAATG | 1780 | |

- 29 -

TGGTTATCGT ATAATGTTAT TACCTCAGTG TCAGGCTGAT GATGTGGGTT CGACTCCCAC 1840
TGACCACTTC AGTTTGAAAT AAGTATTGTC TCGCAACCCT GTTACAGAAT AATTCATTT 1900
ATTACGTGAC AAGATAGTCA TTTATAAAA ATGCACAAAAA ATGTTATTGT CTTTTATTAC 1960
TTGTGAGTTG TAGATTTTC TTATGCGGTG AATCCCCCTT TGCGGCGGGG CGTCCAGTCA 2020
AATAGTTAAT GTTCCTCGCG AACCATATTG ACTGTGGTAT GGTCACCGG GAGGCACCCG 2080
GCACCGCAAT TTTTATAAA ATGAAATTCA CACCTATGG TTCAGAGCGG TGTCTTTTA 2140
CATCAGGTGG GCAAGCATAA TGCAGGTTAA CTTGAAAGAT ACGATCAATA GCAGAAACCA 2200
GTGATTCGT TTATGGCCTG GGGATTTAAC CGCGCCAGAG CGTATGCAAG ACCCTGGCGC 2260
GGTTGGCCGG TGATCGTTCA ATAGTGCGAA TATGAATGGT TACCAAGCCGC CTGCGAATTC 2320

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQUENCE ID NO: 2:

Arg-Gly-Val-Asn-Val-Lys-Tyr-Arg-Tyr-Glu-Asp-Asp-Ser-Phe

- 30 -

Claims

1 1. A method of detecting *Salmonella* in a sample
2 comprising contacting said sample with an antibody
3 specific for a *PhoP* regulated gene product, allowing said
4 antibody to form immune complexes with said *Salmonella*,
5 and detecting said immune complexes as an indication of
6 the presence of *Salmonella* in said sample.

1 2. The method of claim 1, wherein said *PhoP*
2 regulated gene is a *pag*.

1 3. The method of claim 2, wherein said *pag* is
2 *pagC*.

1 4. The method of claim 1, wherein said *PhoP*
2 regulated gene is a *prg*.

1 5. A purified antibody against a *phoP* regulated
2 gene product.

1 6. The purified antibody of claim 5, wherein said
2 antibody is a monoclonal antibody.

1 7. The purified antibody of claim 5, wherein
2 said *phoP* regulated gene is a *pag*.

1 8. The purified antibody of claim 7, wherein said
2 antibody is a monoclonal antibody.

1 9. The purified antibody of claim 7, wherein
2 said *pag* is *pagC*.

1 10. The purified antibody of claim 9, wherein said
2 antibody is a monoclonal antibody.

- 31 -

1 11. The purified antibody of claim 5, wherein
2 said *phoP* regulated gene is a *prg* gene.

1 12. The purified antibody of claim 11, where said
2 antibody is a monoclonal antibody.

1/3

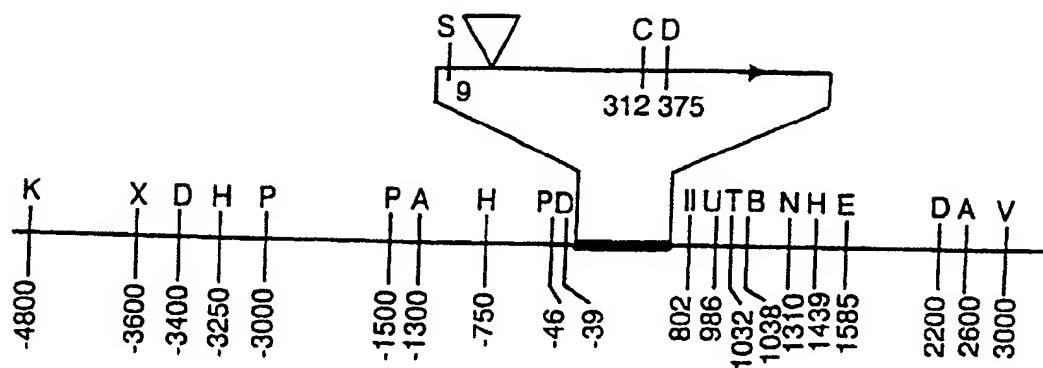


FIG. 1

SUBSTITUTE SHEET

2/3

GTTAACCAC TTTAATAATA ATGGGTTTA TAGCGAAATA CACTTTTTA TCGCGTGTTC 60
 AATATTTGCG TTAGTTATTA TTTTTTGGA ATGTAAATT CTCCTAAACA CAGGTGATAT 120
 TTATGTTGGA ATTGTGGTGT TGATTCTATT CTTATAATAT AACAAGAAAT GTTGTAACTG 180
 * →
 ATAGATATAT TAAAAGATTA AATCGGAGGG GGAATAAACGC GTGCTAAGCA TCATCGTGA 240
 TATGATTACA CGCCCTGCGA TGGCATATAA CCGTATTGCG GATGGAGCGT CACGTGAGGA 300
 CTGTGAAGCA CAATGCGATA TGTTCTGATT ATATGGCGAG TTGCTTAAT GACATGTTT 360
 TAGCCGAACG GTGTCAAAGTT TCTTAATGTG GTTGTGAGAT TTTCTCTTT AATATCAAAA 420
 TGTTGCATGG GTGATTGTT GTTCTATAGT GGCTAAAGAC TTTATGGTTT CTGTTAAATA 480
 TATATGCGTG AGAAAATTA GCATTCAAAT CTATAAAAGT TAGATGACAT TGTAGAACCG 540
 GTTACCTAAA TGAGCGATAG AGTGCTTCGG TAGTAAAAT ATCTTCAGG AAGTAAACAC 600
 ATCAGGAGCG ATAGCGGTGA ATTATCGTG GTTTGTCGA TTCGGCATAG TGGCGATAAC 660
 TGAATGCCGG ATCGGTACTG CAGGTGTTA AACACACCGT AAATAATAAG TAGTATTAAG 720
GAGTTGTT 728
 ATG AAA AAT ATT ATT TTA TCC ACT TTA GTT ATT ACT ACA AGC GTT TTG 776
 Met Lys Asn Ile Ile Leu Ser Thr Leu Val Ile Thr Thr Ser Val Leu
 5 10 15
 GTT GTA AAT GTT GCA CAG GCC GAT ACT AAC GCC TTT TCC GTG GGG TAT 824
 Val Val Asn Val Ala Gln Ala Asp Thr Asn Ala Phe Ser Val Gly Tyr
 20 25 30
 ▼ ▲
 GCA CGG TAT GCA CAA AGT AAA GTT CAG GAT TTC AAA AAT ATC CGA GGG 872
 Ala Arg Tyr Ala Gln Ser Lys Val Gln Asp Phe Lys Asn Ile Arg Gly
 35 40 45
 GTA AAT GTG AAA TAC CGT TAT GAG GAT GAC TCT CCG GTA AGT TTT ATT 920
 Val Asn Val Lys Tyr Arg Tyr Glu Asp Asp Ser Pro Val Ser Phe Ile
 50 55 60
 TCC TCG CTA AGT TAC TTA TAT GGA GAC AGA CAG GCT TCC GGG TCT GTT 968
 Ser Ser Leu Ser Tyr Leu Tyr Gly Asp Arg Gln Ala Ser Gly Ser Val
 65 70 75 80
 GAG CCT GAA GGT ATT CAT TAC CAT GAC AAG TTT GAG GTG AAG TAC GGT 1016
 Glu Pro Glu Gly Ile His Tyr His Asp Lys Phe Glu Val Lys Try Gly
 85 90 95
 TCT TTA ATG GTT GGG CCA GCC TAT CGA TTG TCT GAC AAT TTT TCG TTA 1064
 Ser Leu Met Val Gly Pro Ala Tyr Arg Leu Ser Asp Asn Phe Ser Leu
 100 105 110
 TAC GCG CTG GCG GGT GTC GGC ACG GTA AAG GCG ACA TTT AAA GAA CAT 1112
 Tyr Ala Leu Ala Gly Val Gly Thr Val Lys Ala Thr Phe Lys Glu His
 115 120 125
 TCC ACT CAG GAT GGC GAT TCT TTT TCT AAC AAA ATT TCC TCA AGG AAA 1160
 Ser Thr Gln Asp Gly Asp Ser Phe Ser Asn Lys Ile Ser Ser Arg Lys
 130 135 140

FIG. 2

SUBSTITUTE SHEET

3/3

| | |
|---|-----------|
| ACG GGA TTT GCC TGG GGC GCG GGT GTA CAG ATG AAT CCG CTG GAG AAT Thr Gly Phe Ala Trp Gly Ala Gly Val Gln Met Asn Pro Leu Glu Asn 145 150 155 160 | 1208 |
| ATC GTC GTC GAT GTT GGG TAT GAA GGA AGC AAC ATC TCC TCT ACA AAA Ile Val Val Asp Val Gly Tyr Glu Gly Ser Asn Ile Ser Ser Thr Lys 165 170 175 | 1256 |
| ATA AAC GGC TTC AAC GTC CGG GTT CGA TAC CGT TTC TGA AAAGC Ile Asn Gly Phe Asn Val Gly Val Gly Tyr Arg Phe 180 185 | 1300 |
| | |
| ATAAGCTATG CGGAAGGTTTC GCCTTCCGCA CCGCCAGTCA ATAAAACAGG GCTTCTTAC CAGTGACACCG TACCTGCCTG TCTTTCTCT CTTCGTCATA CTCTCTCGT CATACTGACG | 1360 1420 |
| CTGTACATAA CATCTCACTA GCATAAGCAC AGATAAAGGA TTGTGGTAAG CAATCAAGGT TGCTCAGGTA GGTGATAAGC AGGAAGGAAA ATCTGGTGTAAATAACGCCA GATCTCACAA | 1480 1540 |
| GATTCACTCT GAAAAAATTTT CCTGGAATTAA ATCACAATGT CATCAAGATT TTGTGACCCG CTTCGCATAT TGTACCTGCC GCTGAACGAC TACTGAAAAG TAGCAAGGTA TGTATTTTAT | 1600 1660 |
| CCAGGAGAGC ACCTTTTTG CGCCTGGCAG AAGTCCCCAG CCGCCACTAG CTCAGCTGGA TAGAGCATCA ACCTCCTAAG TTGATGGTGC GAGGTTCGAG GCCTCGGTGG CGGTCCAATG | 1720 1780 |
| TGGTTATCGT ATAATGTTAT TACCTCAGTG TCAGGCTGAT GATGTGGTT CGACTCCCAC TGACCACTTC AGTTTGAAT AAGTATTGTC TCGCAACCT GTTACAGAAT AATTCATT | 1840 1900 |
| ATTACGTGAC AAGATAGTCA TTTATAAAA ATGCACAAAA ATGTTATTGT CTTTTATTAC TTGTGAGTTG TAGATTITTC TTATGCCGTG AATCCCCCTT TGCGGCAGGG CGTCCAGTCA | 1960 2020 |
| AATAGTTAAT GTTCCTCGCG ACCATATTG ACTGTGGTAT GGTTCACCGG GAGGCACCCG GCACCGCAAT TTTTTATAAA ATGAAATTCA CACCTATGG TTCAAGAGCGG TGTCTTTTA | 2080 2140 |
| CATCAGGTGG GCAAGCATAA TGCAAGTTAA CTTGAAAGAT ACGATCAATA GCAGAAACCA GTGATTCGT TTATGCCCTG GGGATTTAAC CGCGCCAGAG CGTATGCAAG ACCCTGGCGC | 2200 2260 |
| GGTTGGCCGG TGATCGTTCA ATAGTGCAGAA TATGAATGGT TACCAAGCCGC CTGCGAATTG | 2320 |

(SEQUENCE ID NO. 1)

FIG. 2a

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02591

| | | |
|--|--|-------------------------------------|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| IPC (5): G01N 33/569; C07K 15/28 US CL : 435/7.35; 530/387.9 | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁴ | | |
| Classification System | Classification Symbols | |
| U.S. | 435/7.35; 530/387.9, 388.2, 388.4, 389.5 | |
| Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵ | | |
| APS, DIALOG, library sequence search; search terms: salmonella and (phoP or pag or pagC), monoclonal or antibod?, author name search | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ | | |
| Category ⁶ | Citation of Document, ¹⁵ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ |
| Y | Trends of Biochemical Sciences, Volume 15, No. 1, issued January 1990, Groisman et al, "Salmonella virulence: new clues to intramacrophage survival", pages 30-33, see final paragraph on page 30. | 1-12 |
| Y | Research in Microbiology, Volume 141, issued 1990, Miller et al, "Salmonella Vaccines with Mutations in the phoP Virulence Regulon", pages 817-821, see last paragraph on page 817. | 1-12 |
| Y | WO, A, 86/01805 (Wright et al) 27 March 1986, see entire document, especially see page 4, lines 5-8 and 28-32 and claims 1 and 34. | 1-12 |
| Y | Proceedings of the National Academy of Sciences, USA, Volume 86, No. 13, issued July 1989, Miller et al, "A two-component regulatory system (phoP phoQ) controls Salmonella typhimurium virulence", pages 5054-5058, especially see Figures 3 and 4. | 1-12 |
| <p>* Special categories of cited documents:¹⁶</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search ² | Date of Mailing of this International Search Report ² | |
| 17 JUNE 1992 | 23 JUN 1992 | |
| International Searching Authority ¹ | Signature of Authorized Officer ²⁰ | |
| ISA/US | CAROL BIDWELL | |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

| | | |
|---|---|------|
| Y | Journal of Bacteriology, Volume 172, No. 5, issued May 1990, Miller et al, "Constitutive Expression of the PhoP Regulon Attenuates <i>Salmonella</i> Virulence and Survival within Macrophages", pages 2485-2490, especially see paragraph bridging pages 2488-2489 and last paragraph on page 2489. | 1-12 |
| Y | Journal of Bacteriology, Volume 173, No. 1, issued January 1991, Pulkkinen et al, "A <i>Salmonella typhimurium</i> Virulence Protein is Similar to a <i>Yersinia enterocolitica</i> Invasion Protein and a Bacteriophage Lambda Outer Membrane Protein", pages 86-93, see page 86, Abstract and paragraph bridging first and second column. | 1-12 |

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. Claim numbers ___, because they relate to subject matter (1) not required to be searched by this Authority, namely:

2. Claim numbers ___, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:

3. Claim numbers ___, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- The additional search fees were accompanied by applicant's protest.
 No protest accompanied the payment of additional search fees.

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|--|-------------------------------------|
| Category* | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁸ |
| A | Infection and Immunity, Volume 58, No. 11, issued November 1990, Miller et al, "Characterization of Defensin Resistance Phenotypes Associated with Mutations in the <i>phoP</i> Virulence Regulon of <i>Salmonella typhimurium</i> " pages 3706-3710. | 1-12 |
| Y | Proceedings of the National Academy of Sciences, USA, Volume 86, No. 18, issued September 1989, Groisman et al, " <i>Salmonella typhimurium phoP</i> virulence gene is a transcriptional regulator", pages 7077-7081, especially see Abstract on page 7077, Figures 2 and 3 and page 7080 paragraph bridging left and right columns. | 1-12 |

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- BLACK BORDERS**
- IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- FADED TEXT OR DRAWING**
- BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- SKEWED/SLANTED IMAGES**
- COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- GRAY SCALE DOCUMENTS**
- LINES OR MARKS ON ORIGINAL DOCUMENT**
- REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.